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# AMYLASES: AN OVERVIEW WITH SPECIAL REFERENCE TO ALPHA AMYLASE

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## INTRODUCTION

Enzymes are biological catalysts which regulate specific biochemical reactions. In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms. Among the industrially important enzymes, proteases and amylases are considered to be the most prominent enzymes since they are widely utilized in brewing, detergent, and food industries[1]. Amylases are employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents. With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many new fields such as clinical, medicinal, and analytical chemistry[2].

Starch processing, which is undertaken in two steps, involves liquefaction of the polysaccharide using bacterial α-amylase, followed by saccharification catalyzed by fungal glucoamylase. After the World War II, enzyme applications increased due to advances in industrial microbiology and biochemical engineering[3]. Now a days, enzymes are employed in many different areas such as food, feed, detergent, textiles, laundry, tanning, as well as pharmaceuticals, cosmetics, and fine-chemicals industries. Industrial applications account for over 80% of the global market of enzymes[4]. At least 50% of the enzymes marketed today are obtained from genetically modified organisms, employing genetic and protein engineering. Food enzymes are the most widely used and still represent the major share in enzyme market.

The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff in wheat and laid down the foundation for the discovery and research on Amylase. The α-amylases were named by Kuhn in 1925, because the hydrolysis products are in the alpha configuration. In 1930, Ohlsson discovered another amylase, which yielded a β-mannose. He named it β-amylase. Crystal structure was established using 3Å resolution structure which was further improved to a 1.5 Å resolution of α-amylases[5]. The three dimensional crystal structures of each form were determined in the 1990s and found to be effectively identical[6].

As diastase, amylase was the first enzyme to be discovered and isolated by Anselme Payen, 1833[7]. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders[8]. Boidin & Effront, 1917 were the first to use *Bacillus. subtilis* and *Bacillus mesentericus* for the production of α-amylases on commercial scale using large fermentors in submerged fermentation[9]. Employment of bacterial cultures for the production of commercial enzyme was pioneered by them and accepted as an industrial practice throughout the world for the production of bacterial α-amylases. Prior to the developments, fungal amylases were extensively produced in the United States by the SSF techniques as pioneered by Takamine[10].

**Types of Amylases**

Enzymes belonging to amylases, endoamylases and exoamylases, are able to hydrolyse starch. These enzymes are classified according to the manner in which the glycosidic bond is attacked. The starch degrading enzymes are found in the numerous glycoside hydrolase families 13(GH-13 families)[11-12].

**α- Amylase (EC 3.2.1.1)**

Endoamylases are able to cleave α,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. α-amylase is a well-known endoamylase. It is found in a wide variety of microorganisms, belonging to the Archaea as well as the bacteria[13]. The end products of α-amylase action are oligosaccharides with varying length with α-configuration and α-limit dextrins, which constitute branched oligosaccharides. α- amylases are often divided into two categories according to the degree of hydrolysis of the substrate[14]. Saccharifying α-amylases hydrolyze 50 to 60% and liquefying α-amylases cleave about 30 to 40% of the glycosidic linkages of starch. The α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium., α-amylase breaks down long-chain carbohydrates by acting at random locations along the starch chain, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. α-amylase tends to be faster-acting than ß-amylase because it can act anywhere on the substrate. In human physiology, both the salivary and pancreatic amylases are α-Amylases and are also found in plants (adequately), fungi (ascomycetes and basidiomycetes) and bacteria (*Bacillus*).

**β- Amylase** (E.C. 3.2.1.2)

Enzymes belonging to the second group, the exoamylases, either exclusively cleave α,1-4 glycosidic bonds such as ß-amylase or cleave both α,1-4 and α,1-6 glycosidic bonds like amyloglucosidase or glucoamylase (E.C. 3.2.1.3) and α-glucosidase (E.C. 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α-glucosidase), or maltose and ß-limit dextrin. ß-amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from α to ß. Glucoamylase and α-glucosidase differ in their substrate preference, α-glucosidase acts best on short maltooligosaccharides and liberates glucose with α-configuration while glucoamylase hydrolyzes long-chain polysaccharides best. β-amylases and glucoamylases have also been found in a large variety of microorganisms[13].

**γ-Amylase (EC 3.2.1.3)**

γ-amylase cleaves α(1-6) glycosidic linkages, in addition to cleaving the last α (1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose. Unlike the other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH of 3.

**Sources of Alfa Amylase**

**Plant sources**

Amylases are widespread in animals, fungi, plants, and are also found in the unicellular eukaryotes, bacteria and archaea[15]. Though plants and animals produce amylases, enzymes from microbial sources are generally used in industrial processes. This is due to a number of factors including productivity, thermostability of the enzyme as well as ease of cultivating microorganisms[16]. The major advantages of the enzymatic route are the selectivity with its associated high yield and exclusivity toward the desired product[17]. Bacteria used in commercial production are the *Bacillus* spp. [18-19, 13]. Others, such as *Escherichia* spp,

*Pseudomonas*, *Proteus*, *Serratia* and *Rhizobium* also yield appreciable quantity of the enzyme [20]. *Aspergillus*, *Rhizopus, Mucor, Neurospora, Penicillium* and *Candida* are some of the fungi that also produce extracellular amylases of commercial value [19]. Plant sources had not been considered with enough significance as the source of these enzymes yet [21]. The utilization of agriculture waste materials serves two functions: reduction in pollution and upgrading of these materials. Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms. These nutrient sources included pearl millet starch, orange waste, potato, corn, tapioca, wheat and rice as flours were used for α-amylase production [22-23]. α-Amylases are being produced commercially in bulk from microorganisms and represent about 25-33% of the world enzyme market[24]. They had numerous applications including liquefaction of starch in the traditional beverages, baking and textile industry for desizing of fabrics [25-26]. Moreover, they have been applied in paper manufacture, medical fields as digestives and as detergent additives [27-28].

**Animal sources**

Ptyalin, a salivary α-amylase (α-1,4-α-D-glucan-4-glucanohydrolase; E.C. 3.2.1.1) is one of the most important enzymes in saliva. The enzyme was first described in saliva by Leuchs in 1831[29]. It consists of two families of isoenzymes, of which one set is glycosylated and the other contains no carbohydrate. The molecular weight of the glycosylated form is about 57 kDa; that of the non-glycosylated form is about 54 kDa. Salivary amylases accounts for 40% to 50% of the total salivary protein and most of the enzyme being synthesized in the parotid gland (80% of the total)[29-30]. It is a calcium-containing metalloenzyme that hydrolyzes the α-1,4 linkages of starch to glucose and maltose. It is known to be mainly involved in the initiation of the digestion of starch in the oral cavity. However, Salivary α-amylase has also been shown to have an important bacterial interactive function[31].

**Microbial sources**

In spite of the wide distribution of amylases, microbial sources, mainly fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and ease of process modification and optimization [32-33]. Fungal amylases have been widely used for the preparation of oriental foods [34]. Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications [35]. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylase.

**Fungal amylases**

Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α-amylase. With the development of genetic engineering, *Bacillus subtilis* is becoming an increasingly attractive host for cloning. The advantages of *B. subtilis* such as high secretion level and non-pathogenic safe (GRAS-generally recognized as safe) status for non-antibiotics strains have made it suitable for the production of heterologous enzymes [36-37]. Most reports about fungi that produce α-amylase have been limited to a few species of mesophilic fungi, and attempts have been made to specify the cultural conditions and to select superior strains of the fungus to produce on a commercial scale [19]. Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* [38]. The *Aspergillus* species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance [39]. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry. *A. oryzae* has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. α-amylase [40]. *Aspergillus oryzae* has been largely used in the production of food such as soy sauce, organic acid such as citric and acetic acids and commercial enzymes including α-amylase

[41]. *Aspergillus niger* has important hydrolytic capacities in the α-amylase production and, due to its tolerance of acidity (pH < 3), it allows the avoidance of bacterial contamination [42]. Filamentous fungi are suitable microorganisms for solid state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate [43]. The fungal α-amylases are preferred over other microbial sources due to their more accepted GRAS status [19]. The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of thrmostable amylase purified the α-amylase, proving its thermostability [44-45].

**Bacterial amylases**

The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermostability. In fact, such industrially important microorganisms found within the genus *Bacillus*, can be exploited commercially due to their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the extracellular medium and safe handling [13].

α-Amylase can be produced by different species of microorganisms, but for commercial applications α-amylase is mainly derived from the genus Bacillus. α-Amylases produced from *Bacillus licheniformis, Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries[46, 13]. Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability. As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C), thermostable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins [47-49]. *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications [50]. Thermostable α-amylases have been reported from several bacterial strains and have been produced using SmF as well as SSF [51].However, the use of SSF has been found to be more advantageous than SmF and allows a cheaper production of enzymes [52]. The production of α-amylase by SSF is limited to the genus Bacillus, and *B. subtilis, B. polymyxia, B. mesentericus, B. vulgarus, B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF [53]. Currently, thermostable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are being used in starch processing industries [48]. Enzymes produced by some halophilic microorganisms have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions [54, 50]. In addition, most halobacterial enzymes are considerably thermotolerant and remain stable at room temperature over long periods. Halophilic amylases have been characterized from halophilic bacteria such as *Chromohalobacter sp*. [50], *Halobacillus sp.* [54], *Haloarcula hispanica* [55], *Halomonas meridian* [56] and *Bacillus dipsosauri* [57].

*Bacillus* is endowed to produce thermostable α-amylase and also large quantities of other enzymes. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus* i.e. *B. subtilis*, *B*. *stearothermophilus*, *B*. *licheniformis* and *B*. *amyloliquefaciens* [33]. Some *Bacillus* strains produce enzyme in the exponential phase, whereas some others in the mid stationary phase. Though, different *Bacillus* species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain. Some properties exhibited by different bactria are shown in following table-

**Table: Bacterial α-amylases & their characteristics**

| Microorganisms | Mode of Fermen-tation | pH optima | Temp. optima | Mol. Wt. (kDa) | Inhibitors | Ref. |
|---|---|---|---|---|---|---|
| *Chromohalobacter* sp. TVSP 101 | SSF | 7.0 - 9.0 | 65 °C | 72 | - | **[50]** |
| *Haloarcula hispânica* | | 6.5 | 50 °C | 43.3 | EDTA | **[50]** |
| *Bacillus* sp. I-3 | SmF | 7.0 | 70 °C | - | EDTA, $HgCl_2$ | **[58]** |
| *Bacillus* sp. PN5 | SmF | 10 | 60 °C | - | $NH_4Cl$ | **[59]** |
| *Bacillus subtilis* DM-03 | SSF | 6.0–10 | 50 °C | - | - | **[60]** |
| *Bacillus subtilis* KCC103 | SmF | 6.5 | 37 °C | - | - | **[61]** |
| *Bacillus* sp. KCA102 | | 7.1 | 57.5 °C | - | - | **[62]** |
| *Bacillus subtilis* JS-2004 | SmF | 7.0 | 50 °C | - | $Co^{2+}$ $Cu^{2+}$ $Hg^{2+}$ $Mg^{2+}$ $Zn^{2+}$ $Ni^{2+}$ $Fe^{2+}$ and $Mn^{2+}$ | **[47]** |
| *Bacillus subtilis* | SmF | 7.0 | 135 °C | - | - | **[46]** |
| *Bacillus caldolyticus* DSM405 | SmF | 5.0-6.0 | 70 °C | - | - | **[63]** |
| *Bacillus* sp. Ferdowsicous | | 4.5 | 70 °C | 53 | $Hg^{2+}Zn^{2+}$ EDTA | **[64]** |
| *Halomonas meridian* | SmF | 7.0 | 37 °C | - | Glu | **[56]** |
| *Geobacillus thermoleovorans* | - | 7.0 | 70 °C | - | - | **[65]** |

**Mode of Action of Alfa Amylase**

In general, it is believed that α-amylases are endo-acting amylases which hydrolyze α- (1-4) glycosidic bonds of the starch polymers internally. Several models for amylase action pattern have been proposed, such as the random action and the multiple attack action. Random action has also been referred to as a single attack or multi-chain attack action **[13]**. In the former, the polymer molecule is successively hydrolysed completely before dissociation of the enzyme-substrate complex. While, in the latter, only one bond is hydrolysed per effective encounter. The multiple attack action is an intermediate between the single-chain and the multi-chain action where the enzyme cleaves several glycosidic bonds successively after the first (random) hydrolytic attack before dissociating from the substrate. It is observed that the multiple attack action is generally an accepted concept to explain the differences in action pattern of amylases **[66]**. However, most of the endoamylases have a low to very low level of multiple attack action increased with temperature to a degree depending on the amylase itself.

**Structure of Alfa Amylase**

**Molecular weight**

Despite wide difference of microbial α-amylases characters, their molecular weights are usually in the same range 40-70 kDa **[19]** while the highest molecular weight of α-amylases, 210 kDa, for *Chloroflexus aurantiacus* **[67]**. Whereas, 10 kDa of *Bacillus caldolyticus* α-amylase was

reported to be the lowest value [19]. This molecular weight may be raised due to glycosylation as in the case of *Thermoactiomyces vulgaris* α-amylase that reach 140 kDa [68]. In contrast, proteolysis may lead to decrease in the molecular weight. For example, α-amylase of *T. vulganis* 94-2A (AmyTV1) is a protein of 53 kDa and smaller peptides of 33 and 18 kDa that have been shown to be products of limited AmyTV1 proteolysis [69].

**Molecular structure**

α-amylases from different organisms share about 30% amino acid sequence identity and all belong to the same Glycosyl Hydrolase family 13 (GH-13 family of protein) [70]. The three dimensional (3D) structures of α-amylases have revealed monomeric, calcium-containing enzymes, with a single polypeptide chain folded into three domains (A-C). The most conserved domain in α-amylase family enzymes, the A-domain, consists of a highly symmetrical fold of eight parallel ß-strands arranged in a barrel encircled by eight α-helices. The highly conserved amino acid residues of the α-amylase family involved in catalysis and substrate binding are located in loops at the C-termini of ß-strands in this domain. This is typical to all enzymes belonging to the α/ß –barrel protein family [71]. α-amylases have a B-domain that protrudes between ß-sheet number 3 and α-helix number. 3. It ranges from 44 to 133 amino acid residues and plays a role in substrate or Ca binding. The sequence of this domain varies most; in *Bacillus,* α-amylases it is relatively long and folds into a more complex structure of ß-strands [72], whereas in barley α-amylase there is an irregularly structured domain of 64 residues. All known α-amylases, with a few exceptions, contain a conserved $Ca^{+2}$ binding site which is located at the interface between domains A and B [73]. In addition, α-amylase produced by *Bacillus thermooleovorans* was found to contain a chloride ion binding site in their active site [74], which has been shown to enhance the catalytic efficiency of the enzyme, presumably by elevating the pKa of the hydrogen-donating residue in the active site [50].

α-amylases has a domain C which is relatively conserved and folds into an antiparallel ß barrel. The orientation of domain C relative to domain A varies depending on the type and source of amylase [75]. The function of this domain is unknown. Structural studies have confirmed that the active sites of glycosyl hydrolases are composed of multiple binding sites, or subsites, for the sugar units of polymeric substrates. The open active site cleft is formed between domains A and B, so that residues from domain B participate in substrate binding. The substrate binding sites are commonly lined with aromatic residues (Phe, Trp and Tyr) which make hydrophobic stacking interactions with the sugar rings. In addition, the active sites contain many residues which form hydrogen bonds to the substrate either directly or via water molecules [76, 66 ]. In Taka-amylase A, the first examined protein α-amylase by X-ray crystallography, three acidic residues, i.e., one glutamic and two aspartic acids were found at the centre of the active site, and subsequent mutational studies have shown that these residues are essential for catalysis [77, 66]. The glutamic acid residue is now believed to be the proton donor, while the first of the two conserved aspartic acids appearing in the amino acid sequence of an α-amylase family member is thought to act as the nucleophile. The role of the second aspartic acid is less certain, but it has been suggested to involved in stabilising the oxocarbenium ion-like transition state and also in maintaining the glutamic acid in the correct state of protonation for activity [78]. These residues occur near the ends of strands 3, 4, 5 and 7 of the a/ß -barrel and are found in four short sequences, long-recognised as being conserved in α-amylase family enzymes.

**Applications of Alfa Amylase**

Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. A large-scale starch processing industry has emerged in the last century. In the past decades, we have seen a shift from the acid hydrolysis of starch to the use of starch-converting enzymes in the production of maltodextrin, modified starches, or glucose and fructose syrups. Currently, these enzymes comprise about 30 % of the world's enzyme production. Besides the use in starch hydrolysis, starch-converting enzymes are also used in a number of other industrial applications, such as laundry and porcelain detergents or as anti-staling agents in baking. A number of these starch-converting enzymes belong to a single family: the alpha amylase family or family13 glycosyl hydrolases. This group of enzymes share a

number of common characteristics such as α (ß/α)8 barrel structure, the hydrolysis or formation of glycosidic bonds in the a conformation, and a number of conserved amino acid residues in the active site. As many as 21 different reaction and product specificities are found in this family.

**Bread and chapatti industry**

The quantities, taste, aroma and porosity of the bread are improved by using the enzyme in the flour. More than 70 % bread in U.S.A, Russia and European countries contain alpha amylase. Amylases play important role in bakery products [79]. For decades, enzymes such as malt and fungal alpha-amylases have been used in bread-making. The significance of enzymes is likely to raise as consumers insist more natural products free of chemical additives. For example, enzymes can be employed to replace potassium bromate, a chemical additive that has been prohibited in a number of countries. The dough for bread, rolls, buns and similar products consists of flour, water, yeast, salt and possibly other ingredients such as sugar and fat. Flour consists of gluten, starch, non-starch polysaccharides, lipids and trace amounts of minerals. As soon as the dough is made, the yeast starts to work on the fermentable sugars, transforming them into alcohol and carbon dioxide, which makes the dough rise. The major component of wheat flour is starch. Amylases can degrade starch and produce small dextrins for the yeast to act upon. The alpha-amylases degrade the damaged starch in wheat flour into small dextrins, which allows yeast to work continuously during dough fermentation,proofing and the early stage of baking. The result is improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions responsible for the browning of the crust and the development of an attractive baked flavor [80].

**Textile industry**

Textile industries are extensively using alpha amylases to hydrolyze and solubilize the starch, which then wash out of the cloth for increasing the stiffness of the finished products. Fabrics are sized with starch. Alpha amylase is used as desizing agent for removing starch from the grey cloth before its further processing in bleaching and dyeing. Many garments especially the ubiquitions 'Jean' are desized after mashing. The desired fabrics are finally laundered and rinsed [81, 82].

**Sugar and glucose industries**

Alpha amylase plays a very important role in the production of starch conversion products of low fermentability. The presence of starch and other polysaccharides in sugar cane creates problem throughout the sugar manufacturing which is minimized or eliminated by the action of alpha amylase. The high quality products depends upon the efficiency of the enzyme which lead to low production, costs for the starch processor has increased [83-85]. Many industries used alpha amylases for the production of glucose. Enzyme hydrolyzed the starch and converted it into glucose. They hydrolyze α-1,4 glucosidic linkage in the starch polymer in a random manner to yield glucose and maltose [86]. Therefore, alpha amylase is extensively used in many industries for the production of glucose and also used in water-soluble dextrin [87].

**Alcohol industry**

Alpha amylases convert starch in to fermentable sugars. Starches such as grain;potatoes etc. are used as a raw material that helps to manufacture ethyl alcohol. In the presence of amylases, the starch is first converted in to fermentable sugars. The use of bacterial enzyme partly replaces malt in brewing industry, thus making the process more economically significant. Alpha amylase can also carries out the reactions of alcoholysis by using methanol as a substrate [88].

**Paper industry**

Starch paste when used as a mounting adhesive modified with additives such as protein glue or alum, frequently, causes damage to paper as a result of its embrittlement. Starch digesting enzymes, e.g. alpha amylase, in immersion or as a gel poultice are applied to facilitate its removal. Alpha amylase hydrolyzed the raw starch that is used for sizing and coating the paper instead of expensive chemically modified starches. So, starch is extensively used for some paper size press publications[89]. Examples of amylases obtained from microorganisms used in

paper industry includes Amizyme® (PMP Fermentation Products, Peoria,USA), Termamyl®, Fungamyl, BAN® (Novozymes,Denmark) and α-amylase G9995® (Enzyme Biosystems, USA) [90].

**Detergent, building product and feed industries**

In detergent industries, the enzyme alpha amylase plays a vital role. It is widely used for improvement of detergency of laundry bleach composition and bleaching with out color darkening [91, 92]. The addition of enzyme stabilizes the bleach agent and preserves effectiveness of the bleach in laundry detergent bar composition [93, 94]. Modified starch is used in the manufacture of gypsum board for dry wall construction. Enzyme modified the starch for the industry use. Many starches or barely material are present in the feed. So, the nutritional value of the feed can be improved by the addition of alpha amylase.

**Chocolate industry**

Amylases are treated with cocoa slurries to produce chocolate syrup, in which chocolate starch is dextrinizing and thus syrup does not become thick. Cocoa flavored syrups having a high cocoa content and excellent stability and flow properties at room emperature may be produced by using an amylolytic enzyme and a sufficient proportion of Dutch process cocoa to provide a syrup pH of 5.5 to 7.5. The syrup is made by alternate addition of cocoa and sweetener to sufficient water to achieve a solids content of about 58 to 65 weight percent, adding an amylolytic enzyme, heating to a temperature of about 175 -185°F for at least 10 to 15 min, raising the temperature to about 200° F. and cooling. The stabilized cocoa flavored syrups may be added at room temperature to conventional non-acid confection mixes for use in the production of quiescently frozen chocolate flavored confections [95].

**Fuel alcohol production**

Ethanol is the most utilized liquid biofuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world [96]. In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as α-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* [97, 98]. The production of ethanol by yeast fermentation plays an important role in the economy of Brazil [99]. In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* [96]. Among bacteria,α-amylase obtained from thermoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions [100].

**Treatment of starch processing waste water (SPW)**

Starch is also present in waste produced from food processing plants. Starch waste causes pollution problems. Biotechnological treatment of food processing waste water can produce valuable products such as microbial biomass protein and also purifies the effluent [101-104].

**Analysis in medicinal and clinical chemistry**

With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry. There are several processes in the medicinal and clinical areas that involve the application of amylases. The application of a liquid stable reagent, based on α-amylase for the Ciba Corning Express clinical chemistry system has been described [105]. A process for the detection of higher oligosaccharides, which involved the application of amylase was also developed [106]. This method was claimed to be more efficient than the silver nitrate test. Biosensors with an electrolyte isolator semiconductor capacitor (EIS-CAP) transducer for process monitoring were also developed [107].

**Other applications**

- An inhibitor of alpha-amylase called phaseolamin has been tested as a potential diet aid [108].
- A higher than normal concentration of amylases may reflect one of several medical conditions, including acute inflammation of the pancreas (concurrently with the more specific lipase), but also perforated peptic ulcer, torsion of an ovarian cyst, strangulation ileus, macroamylasemia and mumps. Amylase may be measured in other body fluids,including urine and peritoneal fluid.
- In molecular biology, the presence of amylase can serve as an additional method of selecting for successful integration of a reporter construct in addition to antibiotic resistance. As reporter genes are flanked by homologous regions of the structural gene for amylase, successful integration will disrupt the amylase gene and prevent starch degradation, which is easily detectible through iodine staining.

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